infected plant galls contain excessive amounts of auxin in comparison with the healthy roots,[6–8] auxin may play an important role in gall formation, and anti-auxin substances might well suppress the disease. This suggests that the clubroot suppression activity of epoxydon is due to the anti-auxin activity.

Recently, *Phoma* spp have been recognized as fungi with plant growth promoting activity (PGPF).[9] The *P glometara* JCM9972 strain seems to be one of them and its application as a biocontrol agent is worth investigation.

P glomerata JCM9972 and epoxydon may constitute a new group of plant protection agents with no fungicidal activity. Hence, this study, while focusing on the control of clubroot, may be much more widely applicable. It is likely that these chemicals could be environmentally compatible and might not produce resistant strains of pathogens.

ACKNOWLEDGEMENTS

This study was partly supported by Special Coordination Funds for promoting Science and Technology of the Science and Technology Agency of the Japanese Government.

REFERENCES

1 Tamura K, Hasegawa Y, Kudo T and Yamaguchi I, Isolation and characterization of the PCNB-degrading bacterium, *Pseudomonas aeruginosa* strain I-41. *Nihon Noyaku Gakkaishi (J Pestic Sci)* **20**:145–151 (1995).

2 Arie T, Kobayashi Y, Okada G, Kono Y and Yamaguchi J, Epoxydon produced by *Phoma glomerata* controls soil-borne clubroot disease of cruciferous plants. *Plant Pathology* (in press).

3 Boerema GH and Dorenbosch MMJ, The *Phoma* and *Ascochyta* species described by Wollenweber and Hochapfel in their study on fruit-rotting. *Studies in Mycology* **3**:1–50 (1973).

4 Closse A, Mauli R and Sigg HP, Die Konstitution von Epoxydon. *Helv Chim Acta* **25**:204–213 (1966).

5 Sakai R, Sato R, Niki H and Sakamura S, Biological activity of phyllosinol, a phytotoxic compound isolated from a culture filtrate of *Phyllosticta* sp. *Plant and Cell Physiology* **11**:907–920 (1970).

6 Raa J, Indole-3-acetic acid levels and the role of indole-3-acetic oxidase in the normal root and club root of cabbage. *Physiologia plantarum* **25**:130–134 (1971).

7 Ludwig-Mueller J, Bendel U, Thermann P, Ruppel M, Epstein E and Hilgenberg W, Concentrations of indole-3-acetic acid in plants of tolerant and susceptible varieties of Chinese cabbage infected with *Plasmodiophora brassicae* Woron. *New Phytologist* **125**:163–169 (1993).

8 Bischoff M, Low R, Grsic S, Rausch T, Hilgenberg W and Ludwig-Mueller J, Infection with the obligate biotroph *Plasmodiphora brassicae*, the causal agent of the clubroot disease, does not affect expression of NIT 1/2-related nitrilases in root of Chinese cabbage. *J Plant Physiol* **147**:341–345 (1995).

9 Shibata N, Kageyama K, Hyakumachi M, Sasaki C, Yoshida R and Sato Y, Production of abscisic acid by plant growth-promoting fungi. *Ann Phytopathol Soc Jpn* **63**:237 (1997).

Ion regulation in the larval lepidopteran midgut and the response to *Bacillus thuringiensis* δ-endotoxin

J Lawrence Gringorten*
Candian Forest Service, 1219 Queen St E, PO Box 490, Sault Ste. Marie, ON P6A 5M7, Canada

Abstract: Fourth-instar *Bombyx mori* (silkworm) was used as a model insect to study the effects of *Bacillus thuringiensis* (Bt) on ion homeostasis in the larval lepidopteran midgut. The K^+ chemical gradient across the midgut of *B. mori* larvae is quite small, sustained by a lumen/haemolymph activity ratio of only 1.6. More than 95% of the driving force causing K^+ flux from lumen to haemolymph is electrical. In contrast to K^+, the H^+ chemical gradient is exceedingly large, with luminal pH values of 11–12 and haemolymph/lumen H^+ ratios as high as 10^5. At equilibrium, the steep proton gradient is consistent with a passive distribution of H^+ across the midgut epithelium as predicted from the Nernst equation. In *B mori* larvae, ingestion of a lethal dose of Bt δ-endotoxin produces an increase in K^+ conductance in the midgut apical epithelium, causing a decrease in the electrical gradient and dissipation of the pH gradient. Larval morbidity can be correlated with a rise in haemolymph K^+ and pH and a decline in luminal pH. Midgut K^+ activity, however, remains unchanged. An important factor in the pathogenesis of Bt is irreversible alkalization of the epithelial cells as H^+ is redistributed across the midgut to reach a new Nernst equilibrium.

Keywords: *Bacillus thuringiensis*; Lepidoptera; midgut; ion regulation; potassium; pH

1 INTRODUCTION

The pH of the larval lepidopteran midgut is one of the highest known for any biological system. Values in the range pH 10–11, and at times as high as pH 12, have been recorded in *Bombyx mori* L and other species.[1] This extreme alkalinity is thought to be an evolutionary adaptation to a tannin-rich leaf diet, and is mediated by secondary active transport of K^+ into the midgut lumen by goblet cells. The present model for this process is based on an ATP-dependent, electrogenic, primary H^+ pump coupled with an electrophoretic $1K^+/2H^+$ antiporter in the goblet cell apical membrane (GCAM).[2] The H^+ pump is a vacuolar-type ATPase visible as portasomes (10-nm particles) lining the cytoplasmic side of the GCAM.[3] The exchange of H^+ for K^+ by the antiporter results in K^+, rather than H^+, being pumped into the midgut lumen, allowing for alkalization, rather than acidification, of the luminal contents. Overall electroneutrality is maintained by secretion of carbonate into the lumen

* Correspondence to: J Lawrence Gringorten, Canadian Forest Service, 1219 Queen St E, PO Box 490, Sault Ste Marie, ON P6A 5M7, Canada
E-mail: lgringo@nrcan.gc.ca
(*Received 20 July 1998; accepted 5 January 1999*)

	pH		*K^+ (mM)*		*Na^+ (mM)*[b]	
	Midgut	*Haemol*	*Midgut*	*Haemol*	*Midgut*	*Haemol*
Control	11.2 (+0.1)	6.6 (+0.1)	92 (+8)	59 (+4)	<0.1	3
Bt treated	8.4 (+0.2)	7.7 (+0.3)	98 (+13)	96 (+23)	0.3	1

Table 1. Ion activities in the midgut and haemolymph of *Bombyx mori* force-fed a lethal dose of Bt activated toxin.[a]

[a] Means (+ 95% CL).
[b] Na^+ sample size was too small for confidence limits.

by the epithelial cells, leading to the formation of potassium carbonate, a highly alkaline compound.[3,4]

The H^+–K^+-coupled pump system charges the apical membrane and generates a high electrical potential difference (PD) between lumen and haemolymph (lumen positive). The PD provides a driving force for H^+ flux from lumen to haemolymph and the development of a steep H^+ chemical gradient across the midgut, with the lumen normally at pH 10–12 and the haemolymph at less than pH 7. At equilibrium, this gradient is consistent with a passive distribution of H^+ across the midgut epithelium as predicted from the Nernst equation.

The high midgut pH is a critical factor in the pathogenesis of *Bacillus thuringiensis* Berliner (Bt). Bt δ-endotoxins are synthesized as parasporal crystal proteins and are protoxins of molecular mass approximately 120 kDa, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).[5] The high pH and reducing conditions of gut juice are necessary for their solubilization *in vivo*, following which, midgut enzymes effect their proteolytic conversion to 60–65 kDa active toxins. In susceptible insects the toxins exert their insecticidal action by causing alkaline and osmotic lysis of the midgut epithelial cells. In this summary, using *B mori* as a model, K^+ and H^+ homeostasis in the larval lepidopteran midgut and the disruption caused by Bt δ-endotoxins are described.

2 EXPERIMENTAL

Semi-microelectrodes were used to measure ion activities in the midgut lumen and haemolymph of fourth-instar *B mori* larvae. Bt subsp *kurstaki* strain HD-1 δ-endotoxin was extracted in 0.1 M CAPS-KOH buffer, pH 10.5, and activated in 1% *B. mori* larval gut juice. Total protein concentration was determined by the Coomassie Blue dye-binding method[6] and quantification of the 60–65 kDa active ingredient performed by SDS-PAGE[7] and scanning densitometry. Fourth-instar silkworms were force-fed activated toxin with the aid of an electronic microinjector.

3 RESULTS AND DISCUSSION

K^+ and H^+ homeostasis are intricately linked and support a number of secondary processes upon which the viability of the larva depends. In live *B mori* larvae, the PD between lumen and haemolymph, generated

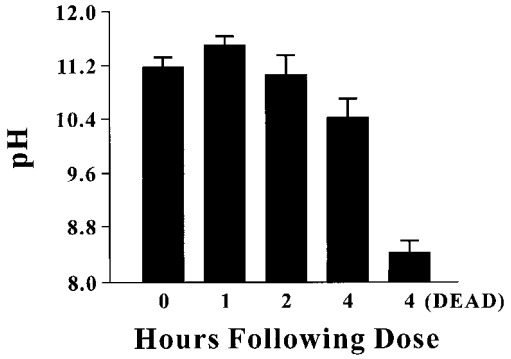

Figure 1. Time-course changes in midgut pH in *Bombyx mori* larvae force-fed Bt activated toxin. The dose caused 67% mortality in a subgroup of insects. Bar = 95% CL.

by the secondary K^+ pump in the GCAM, has been measured at over 300 mV (lumen positive). At this potential, the Nernst equation predicts a pH differential of about 5 units across the midgut, if proton distribution is passive. This is, in fact, within the range observed for *B mori* and establishes an extraordinary chemical gradient for H^+, with lumen pH at 11–12, haemolymph pH at less than 7 and the haemolymph/lumen H^+ activity ratio as high as 10^5.

In contrast, the chemical gradient for K^+ across the midgut of *B mori* is very small, sustained by a lumen/haemolymph ratio of only 1.6, and represents less than 5% of the total electrochemical gradient down which K^+ moves from the lumen into the haemolymph. Most –more than 95% – of the driving force that cycles pumped K^+ back to the haemolymph is electrical, produced by the secondary K^+ pump itself. The high pH of the midgut promotes binding of dietary K^+ to proteins,[8] keeping its activity in the midgut and the chemical gradient between lumen and haemolymph relatively low.

In addition to its key role in the activation of Bt δ-endotoxin, the high pH of the midgut is also important in maximizing the cytolytic effect.[9] The initial response to Bt when ingested by a susceptible insect is cessation of feeding. At the molecular level, there is an increase in K^+ conductance across the apical epithelium of the midgut, which causes the transmembrane PD to fall.[10] It collapses completely when a lethal dose is ingested, which in turn causes the pH gradient to dissipate as H^+ ions redistribute themselves and reach a new Nernst equilibrium. This results in acidification of the midgut lumen and alkalization of the epithelial cells and haemolymph (Table 1). The decline in midgut pH is not immediate.

A close examination of the time course in *B mori* reveals that, prior to the decrease the pH peaks (Fig 1) The mechanism mediating this rise is unknown, but may be a non-specific stress response to the force-feed procedure.

Luminal K^+ activity is largely unaffected by Bt intoxication, but K^+ activity in the haemolymph increases by more than 60%, abolishing even the small chemical gradient that existed under equilibrium conditions (Table 1). The source of the additional haemolymph K^+ is uncertain, but may well be the midgut lumen itself. As the transmidgut PD decreases and luminal contents become acidified, it is possible that protein-bound K^+ is released[8] and driven toward the haemolymph side until the activities in both compartments become equal.

Irreversible destruction of the midgut epithelium by Bt δ-endotoxins is caused by a combination of cell alkalization[10] and colloid osmotic lysis.[11] Most insects susceptible to Bt, however, do not succumb directly to the endotoxin itself, but to the septicemia that follows, either from germinating Bt spores in the gut, if challenged with spore-crystal mixtures, or from other opportunistic pathogens that may be present in the gut.

REFERENCES

1 Gringorten JL, Crawford DN and Harvey WR, High pH in the ectoperitrophic space of the larval lepidopteran midgut. *J Exp Biol* **183**:353–359 (1993).

2 Wieczorek H, Putzenlechner M, Zeiske W and Klein U, A vacuolar-type proton pump energizes K^+/H^+ antiport in an animal plasma membrane. *J Biol Chem*, **266**:15340–15347 (1991).

3 Harvey WR, Physiology of V-ATPases. *J. Exp Biol*, **172**:1–17 (1992).

4 Dow JAT, Extremely high pH in biological systems: a model for carbonate transport. *Am J Physiol* **246**:R633–R635 (1984).

5 Fast PG, The crystal toxin of *Bacillus thuringiensis*, in *Microbial Control of Pests and Plant Diseases*. ed by Burges HD, Academic Press, New York and London. pp 223–248 (1981).

6 Bradford MM, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, **72**:248–254 (1976).

7 Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680–685 (1970).

8 Dow JAT and Harvey WR, Role of midgut electrogenic K^+ pump potential difference in regulating lumen K^+ and pH in larval Lepidoptera. *J Exp Biol* **140**:455–463 (1988).

9 Gringorten JL, Milne RE, Fast PG, Sohi SS and Frankenhuyzen K, Suppression of *Bacillus thuringiensis* δ-endotoxin activity by low alkaline pH. *J Invert Pathol* **60**:47–52 (1992).

10 Harvey WR, Cioffi M and Wolfersberger MG, Transport physiology of lepidopteran midgut in relation to the action of Bt delta-endotoxin, in *Fundamental and Applied Aspects of Invertebrate Pathology*, ed by Samson RA, Vlak JM and Peters D, Foundation of the Fourth International Colloquium of Invertebrate Pathology, Wageningen. pp 11–15 (1986).

11 Knowles BH and Ellar DJ, Colloid-osmotic lysis is a general feature of the mechanism of action of *Bacillus thuringiensis* δ-endotoxins with different insect specificity. *Biochim Biophys Acta* **924**:509–518 (1987).

Molecular detection of insecticide resistant alleles

J Marshall Clark,* A Zhang, J Dunn and K Yoon
Department of Entomology, University of Massachusetts, Amherst, MA 01003-2410, USA

Abstract: The summary discusses techniques used to investigate mechanisms of resistance in the Colorado potato beetle (*Leptinotarsa decemlineata*) to several classes of pesticides.

Keywords: Colorado potato beetle; resistance; mutation; single-stranded polymorphism; PCR amplification of specific alleles; DNA mini-sequencing

The Colorado potato beetle (CPB) (*Leptinotarsa decemlineata* (Say)) is a world-wide pest of potatoes and is resistant to all major classes of insecticides. Detailed biochemical and pharmacological studies have established a variety of resistance mechanisms; including altered acetylcholinesterases (organophosphates and carbamates),[1] insensitive ion channels (DDT and pyrethroids),[2] sequestration proteins associated with the hemolymph (multiple insecticides)[3] and induced oxidative metabolism (abamectin).[4] We have devised a number of molecular-based diagnostic procedures to detect mutations and over-expressed proteins that result in resistance, including single-stranded conformational polymorphism (SSCP), PCR amplification of specific alleles (PASA) and mini-sequencing of DNA coupled with immunochemical detection.

A point mutation in the acetylcholinesterase (AChE) gene associated with azinphos-methyl resistance

We have cloned and sequenced a cDNA encoding the AChE of azinphosmethyl-susceptible (SS) strain of CPB.[1] The deduced amino acid sequence consisted of 29 residues for the putative signal peptide and 600 residues for the mature protein. A point mutation (A→G, nt location 980) that resulted in a Ser (AGT)/Gly (GGT) amino acid change (designated [S291G])[5] was identified in the azinphos-methyl-resistant strain of CPB (AZ-R). The A→G mutation was found in all AChE cDNA sequences amplified by PCR from enzymatically authenticated AZ-R beetles but was never found in AChE cDNA sequences from SS beetles.[5] The Ser/Gly change, however, does not occur within either the esteratic subsite or the peripheral anionic site of AChE. This amino acid residue corresponds to Val 238 of the *Torpedo* AChE and represents the first residue to form the α-helix, $\alpha\text{-E}'_1$. The predicted secondary structure of the mutation-containing region of the AZ-R AChE indicates that the transition from the turn sequence to the α-helix sequence occurs sooner in the sequence when Ser is replaced by Gly. Thus, the Ser/Gly change is

* Correspondence to: J Marshall Clark, Department of Entomology, University of Massachusetts, Amherst, MA 01003-2410, USA
E-mail: Jclark@ent.umass.edu

(*Received 16 July 1998; accepted 5 January 1999*)